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Genetic variation at chloroplast microsatellites (cpSSRs) in *Abies nebrodensis* (Lojac.) Mattei and three neighboring *Abies* species

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Abstract *Abies nebrodensis* (Lojac.) Mattei (Sicilian fir) is an endangered species represented by only one population of 29 adult individuals occurring in a limited area of the Madonie Range in northern Sicily (Italy). Taxonomic boundaries between this taxon and the neighboring *Abies* species are not clear. In this study, we used six chloroplast simple-sequence repeats (cpSSRs) to investigate the population genetic structure and the distribution of chloroplast haplotypic variation in *A. nebrodensis* and three of the neighboring *Abies* species: *Abies alba* (Mill.), *Abies numidica* (De Lann) and *Abies cephalonica* (Loud.). Our aims were to quantify the level of cpDNA differentiation within the *Abies* populations and to shed light on the history of *A. nebrodensis.* Diversity levels based on the haplotype frequency at six cpSSRs were high, especially in *A. alba* and *A. cephalonica*. In all, we found 122 haplotypes among the 169 individuals analyzed, and the four species were distinguished from each other by their haplotype composition. The majority of the haplotypes (76%) were detected only once, but in *A. nebrodensis* seven individuals (41% of the sample population) shared the same haplotype. Moreover, the seven *A. nebrodensis* individuals with an identical haplotype showed a tendency to be geographically grouped within the population's limited range. The analysis of molecular variance (AMOVA) showed a significant difference in the level of apportionment of gene diversity between the species *A. alba* and *A. cephalonica* (F_{ST}=0.191 and 0.012, respectively). AMOVA analysis conducted over all populations from the four species showed that 19% of the total cpSSR variation was attributable to differences among species, 6% was due to differences among populations within species, and 74% to differences within populations. The high percentage of unique haplotypes identified confirms the power of cpSSR haplotype analysis for identifying individual trees in individual *Abies* populations. Our results indicate that *A. nebrodensis* differs from the other three *Abies* species investigated and support its classification as an independent taxon. The results also showed a decreased level of variation in *A. nebrodensis* and suggested that the species has experienced a genetic bottleneck during the last two centuries.

Keywords *Abies nebrodensis* · Sicilian fir · Population genetics · Chloroplast microsatellites · Simple-sequence repeats

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Introduction

The genus *Abies* is complex in comparison with other genera of the family *Pinaceae*. It includes 49 different species widely distributed over the Northern Hemisphere and with a great variability in their morphological traits (Farjon and Rushforth 1989). Because of this great variability, the taxonomy of the genus has been in a state of confusion for many years.

The status of *Abies nebrodensis* (Lojac.) Mattei (Sicilian fir) is one among the many unresolved taxonomic issues concerning this genus. According to Morandini et al. (1994) the species was once widely distributed in the Madonie Range of northern Sicily (Italy), but over the last two centuries was almost completely destroyed by man. Now it is extremely rare and represented by only 29 adult individuals endemic to a limited area of the Madonie Range, which has been declared a natural reserve. The main questions relating to *A. nebrodensis* are its unknown origin and its uncertain taxonomic position. According to many authors (Nitzelius 1969; Liu 1971; Morandini et al. 1994) the individuals from

A. nebrodensis share morphological similarities with three neighboring *Abies* species, namely *Abies alba* (Mill.) (Silver fir), *Abies numidica* (De Lann.) (Algerian fir) and *Abies cephalonica* (Loud.) (Greek fir). The range of *A. alba* is the widest of all the European *Abies* species and it extends over the mountainous regions of Italy, central Europe and the Balkan Peninsula. *A. cephalonica* is a native of the mountains of central and southern Greece, as well as growing on the islands of Euboea and Cephalonia. Finally, *A. numidica* has a very limited range restricted to Mount Babor in the Kabilye Range of northern Algeria.

Taxonomists do not agree about the classification of *A. nebrodensis*. According to Liu (1971) and Farjon and Rushforth (1989) this taxon should be classed as an independent species, while other authors have classified it as a subspecies of *A. alba* (Franco 1950; Nitzelius 1969; Landry 1984). However, relying upon morphological differences for distinguishing among plant species may be difficult even for experts. Recent methods based on molecular markers can be used to clarify the taxonomy of species with uncertain origin (Bousquet et al. 1990; Bacilieri et al. 1996; Watano et al. 1996).

Vicario et al. (1995) employed allozymes, in conjunction with chloroplast DNA (cpDNA) and RAPD markers, to assess the genetic relationships between seven Italian populations of *A. alba* and the population of *A. nebrodensis*. Their results showed clear differences between the two taxa, although they did not detect any differences at the cpDNA level. Using PCR-RFLP analysis of cpDNA in ten *Abies* species, Parducci and Szmidt (1999) recently found that *A. nebrodensis* differs from the other nine taxa studied, including *A. alba* and *A. cephalonica*, while it shared one haplotype with *A. numidica*.

Recently, the analysis of hypervariable simple-sequence repeats (microsatellites or SSRs) (Tautz 1989; Powell et al. 1996) has aroused considerable interest in the population genetic analysis of forest-tree species. The occurrence of SSR polymorphism in the chloroplast genome of pines has been well documented (Powell et al. 1995; Vendramin et al. 1996). Chloroplast SSR markers (cpSSRs) provide a valuable means to study the genome organization, as well as to quantify the level of variation, in the chloroplast genome of many conifer species (Cato and Richardson 1996; Bucci et al. 1998; Morgante et al. 1998) including the genus *Abies* (Vendramin et al. 1999). As a result of their uni-parental inheritance, cpSSRs are expected to show pronounced levels of population differentiation (Ennos 1994; Powell et al. 1995; Vendramin et al. 1999). Moreover, DNA markers for uni-parentally inherited genomes are more-sensitive indicators of reductions in population size than diploid nuclear genomes in monoecious species, because they have only half the effective population size (Birky 1988) and are very useful, therefore, for detecting populations that have passed through genetic bottlenecks (Morgante et al.1998).

In the study presented here, we have used six chloroplast microsatellites to investigate the population genetic structure and the distribution of chloroplast haplotypic variation in *A. nebrodensis* and three of the neighboring *Abies* species: *A. alba* (Mill.), *A. numidica* (De Lann) and *A. cephalonica* (Loud.). Our aims were to quantify the level of cpDNA differentiation within the *Abies* populations and to shed light on the history of *A. nebrodensis*.

Material and methods

Material

Eight natural populations of the species *A. alba*, *A. cephalonica*, *A. nebrodensis* and *A. numidica* were investigated in this study. Table 1 gives the names, provenances, sample sizes, plant material and sampling strategy for the eight populations, all of which were analyzed at six cpSSR loci. Depending on the material available, buds, needles or endosperms from adult trees or seedlings were used for the analysis (see Table 1). It is important to emphasize that chloroplast DNA is present in the embryo, as well as in the endosperm material, of the *Abies* seeds (Ziegenhagen et al.1996).

For *A. alba*, since genetic variation at two of the six cpSSR loci used in the study (Pt30204 and Pt71936) was recently analyzed in multiple populations sampled all over the species range by Vendramin et al. (1999), we collected material from only two populations representing two different regions of the range (Calabria in southern Italy and central Germany). For *A. cephalonica* we collected material from four populations. In particular, samples from population CEPE were collected in central Greece where the range of *A. cephalonica* and *A. alba* overlap. According to some authors, the *Abies* populations growing in this region share morphological characteristics with both of the species and belong to the hybrid species *Abies borisii-regis* (*A. alba*×*A. cephalonica*) (Fady and Conkle 1993; Scaltsoyiannes et al. 1999). Finally, for *A. numidica*, with a limited geographical distribution, we sampled one population representative of the whole range.

Figure 1 shows the exact location of all the 29 adult individuals of *A. nebrodensis* growing on the Madonie Range in Sicily. Triangles and circles indicate the 19 individuals analyzed in this study.

CpSSR analysis

The method used for DNA extraction is described by Doyle and Doyle (1990). Six primer pairs based on sequences of the *Pinus thunbergii* chloroplast genome (Wakasugi et al. 1994) were employed to amplify six chloroplast DNA regions containing short stretches of simple mononucleotide repeats. The primers used were Pt15169, Pt30204, Pt63718, Pt71936, Pt109567 and Pt110048 (Vendramin et al. 1996). PCR-amplification and sizing of the amplification products was performed as described by Vendramin and Ziegenhagen (1997), using an automated laser fluorescence (ALF) DNA sequencer (Pharmacia). Sizing was repeated at least two-times to confirm the fragment lengths obtained. Fragment sizes expressed in base pairs (bp), were calculated using the computer program FRAGMENT MANAGER version 1.2 (Pharmacia) by comparison with internal and external standards.

For ease of presentation, we will use the terms “locus” to refer to a cpSSR site, and “allele” to refer to a size-variant at a given cpSSR site. Since the chloroplast genome does not recombine, it may be viewed as a single locus, and all of the alleles detected as giving rise to different haplotypes of the genome. Therefore, in this study, standard measures of genetic diversity were used to characterize haplotype variation in the analyzed populations. For recording haplotype frequencies, each unique combination of cpSSR alleles across the six cpSSR loci was scored as a different haplotype.

We calculated chloroplast haplotype variation within populations by estimating the total number of haplotypes (*No.*), the effective number of haplotypes (N_e), as well as the unbiased haplotype diversity (H_e) (Nei 1978):

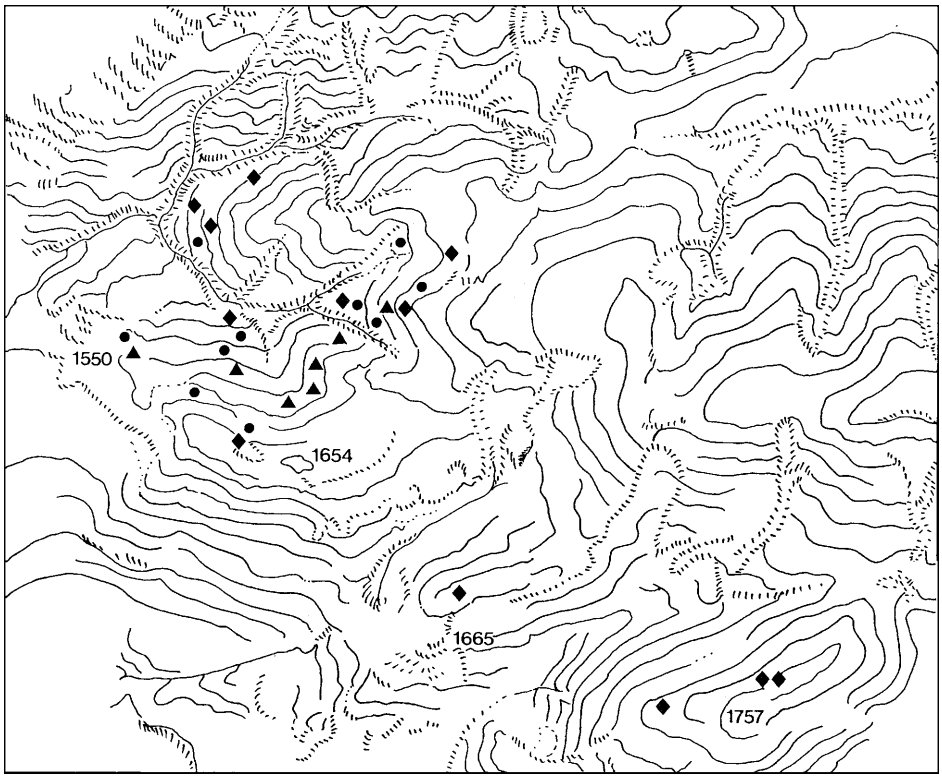

Fig. 1 Location of the 29 individuals of the *A. nebrodensis* population in the Madonie Range of Sicily (scale 1:15000). (•) Individuals with a unique haplotype; (▲) individuals sharing the same haplotype; (♦) individuals not analyzed in the study

$$N_e = \frac{1}{\sum_{i=1}^{m} p^2},$$

$$H_e = \left[\frac{n}{n-1}\left(1 - \sum_{i=1}^{m} p_i^2\right)\right]$$

where n is the total number of individuals analyzed, p is the relative frequency of the *i-th* haplotype and m is the number of haplotypes detected within a population. We also calculated an additional measure of haplotype variation called "proportion distinguishable" (Ellstrand and Roose 1987), which is the ratio of genets (in this case haplotypes) relative to the total number of individuals analyzed in the population.

Genetic distances among individuals within populations were estimated using the D^2_{sh} value based on the stepwise mutation model (Goldstein et al. 1995) adjusted for haplotypic data (Morgante et al. 1998; Bucci and Vendramin, unpublished results) according to the formula:

$$\overline{D^2_{sh}} = \frac{1}{[n\cdot(n-1)]/2} \cdot \frac{1}{L} \cdot \sum_{i=1}^{n} \sum_{i'=(i+1)}^{n} \left[\sum_{k=1}^{L} \left|a_{ik} - a_{i'k}\right|\right]^2$$

where n is the total number of individuals analyzed, L is the number of loci, a_{ik} is the size in bp of the allele at the *i-th* individual and at the *k-th* locus, $a_{i'k}$ is the size in bp of the allele of the *i'-th* individual at the *k-th* locus, and $|a_{ik}–a_{i'k}|$ is the absolute difference in bp between the allele sizes of the two considered individuals.

Apportionment of genetic variation within and among populations was determined by the analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using the Arlequin program 1.1 (Schneider et al. 1997). The significance of the fixation indices was tested using the non-parametric permutation approach described in Excoffier et al. (1992), with 1000 permutations. Distances between cpSSR haplotypes were calculated from the number of different alleles between two haplotypes. When used for estimating genetic structure, this distance will lead to a weighted average F_{ST} over loci, as described by Weir and Cockerham (1984).

Results

Five of the six cpSSR loci analyzed (Pt15169, Pt30204, Pt63718, Pt71936 and Pt110048) were polymorphic in all the eight *Abies* populations investigated, giving a total of 42 different cpSSR alleles among 169 individuals. We found a single allele at locus Pt109567, while locus Pt30204 contributed most to the differentiation among populations, with 18 different alleles. Of the 42 alleles detected, nine were unique to a particular population (Table 1); however, only one allele (142 bp) at locus Pt30204 in *A. numidica* was considered as specific for this species since it occurred with a frequency of 0.50.

When alleles at each of the six loci were jointly analyzed, a total of 122 different haplotypes were identified among the 169 individuals. The majority of these haplotypes (76%) were detected only once, 15% were detected twice and the rest were found in 3–5 individuals, except for one haplotype found in seven individuals of *A. nebrodensis* (41% of the sample population).

Table 1 gives the derived estimates of chloroplast haplotype variation based on six cpSSR loci in the eight *Abies* populations investigated. The lowest value of haplotype diversity was observed in *A. nebrodensis* (H_e=0.846) and the highest (H_e=1.000) in two populations of *A. cephalonica* (CETA and CEPE). Estimates of the effective number of haplotypes and haplotype diversity averaged across all the populations were 14.9 and 0.964, respectively. The estimate of the total cpSSR variation calculated over all populations was 0.996. Values of mean pairwise distance within populations (D^2_{sh}) varied greatly

Table 1 Names, provenances, sample sizes, plant material and sampling strategy for the eight *Abies* population analyzed at six cpSSR loci. (*No*.) total number of haplotypes, (N_e) effective number of haplotypes, (H_e) unbiased haplotype diversity (Nei 1978), (D^2_{sh}) mean distance of individuals within populations (Goldstein et al. 1995), "proportion distinguishable" (Ellstrand and Roose 1987) and frequencies of unique alleles

Taxa	Provenance		Sample size	Plant material, sampling strategy	*No.*	N_e	H_e	D^2_{sh}	Portion distinguishable (%)	Frequencies of unique alleles
ALBA-1 *A. alba*	Germany, Trippstadt	49°20´N 8°50´E	23	Needles from adult trees	12	9.4	0.933	19.7	52.2	(Pt63718:98) 0.043
ALBA-2 *A. alba*	Italy, Calabria, Sottopiano	39°95´N 16°18´E	22	Needles from 10 year-old seedlings	19	17.6	0.991	6.6	86.4	(Pt15169:156) 0.045
NEBR *A. nebrodensis*	Italy, Sicily Mt. Scalone	37°51´N 14°2´E	17	Buds from adult trees	11	4.9	0.846	5.1	64.7	
NUM *A. numidica*	Algeria, Mt. Babour	36°30´N 5°51´E	28	Endosperms from a single-tree collection	18	15.2	0.968	1.8	64.2	(Pt30204:142) 0.500
CEKE *A. cephalonica*	Greece, Cephalonia Island, Mt. Ainos	38°08´N 20°41´E	22	Endosperms from a single-tree collection	18	18.1	0.978	7.5	81.8	(Pt63718:10) 0.045
CEXY *A. cephalonica*	Greece, Helidorea Mt. Killini	38°01´N 22°22´E	24	Endosperms from a single-tree collection	22	20.6	0.993	26.3	91.2	(Pt15169:171) 0.125 (Pt30204:156) 0.042
CETA *A. cephalonica*	Greece, Taygetos Mt. Taygetos	37°16´N 22°18´E	17	Endosperms from a single-tree collection	17	17	1.000	15.9	100	(Pt30204:139) 0.058 (Pt30204:160) 0.058
CEPE *A. cephalonica*	Greece, Pertouli Mt. Pindos	39°10´N 21°35´E	16	Endosperms from a single-tree collection	16	16	1.000	5.2	100	(Pt30204:153) 0.062
Average			21		17	14.9	0.964	11	80	
Total			169		122		0.996			

Table 2 Frequencies of the nine shared haplotypes based on six cpSSR loci in the eight *Abies* populations investigated

Item	h_{40}	h_{46}	h_{75}	h_{73}	h_{74}	h_{96}	h_{77}	h_{49}	h_{26}	%
ALBA-1										0
ALBA-2	0.090	0.045	0.045							15.7
NEBR										0
NUM	0.107									5.5
CEKE					0.045	0.045	0.045			16.6
CEXY					0.083	0.041		0.041	0.041	18.2
CETA				0.058			0.058		0.058	17.6
CEPE		0.062	0.062	0.062	0.062	0.062		0.062		37.5
Average										13.9

Table 3 Analysis of molecular variance (AMOVA) based on six cpSSR loci in the eight *Abies* populations investigated

Item	*df*	Variance components	% Variation	*P*
• Between two *A. alba* populations	1	0.310	19.13	0.000
• Among four *A. cephalonica* populations	3	0.016	1.25	0.143
• Among eight *Abies* populations				
– Among species	3	0.318	19.23	0.010
– Among populations within species	4	0.099	6.10	0.000
– Within populations	161	1.218	74.66	0.000

among populations, from a minimum of 1.8 in *A. numidica* to 26.3 in population CEXY. The "proportion distinguishable" was high and ranged between 52% in population ALBA-1, to 100% in populations CETA and CEPE, where all individuals had unique haplotypes.

The majority of the haplotypes detected in the study (92%) were unique to a particular population. However, with the exception of the haplotype detected in seven individuals of *A. nebrodensis*, they all were observed in one or a few individuals in single populations, which suggests that their low frequency is the result of the limited sample size and that they probably are not true population markers.

We found nine haplotypes common to more than one of the eight populations analyzed, only three of which were shared among different species. The frequencies of the nine haplotypes in each population are given in Table 2. No haplotypes were found in common between the two *A. alba* populations, while six haplotypes were found in common amongst the four *A. cephalonica* populations. In particular, two of the haplotypes detected in population CEPE were also observed in population ALBA-2. The latter population also shared one haplotype with *A. numidica*, while population ALBA-1 and *A. nebrodensis* did not share any haplotype with the other *Abies* populations investigated.

AMOVA analysis showed a significant difference in the apportionment of genetic diversity between the species *A. alba* and *A. cephalonica* (Table 3). In *A. alba* more than 19% of the cpSSR variation detected was attributed to differences among the two populations analyzed (F_{ST}=0.191, *P*=0.000). In contrast, in *A. cephalonica* 98% of the cpSSR variation was due to the within-population components while the percentage of variation due to differences among populations was not significantly different from zero (F_{ST}=0.012, *P*=0.143). AMOVA analysis conducted among all eight *Abies* populations showed that 19% of the total cpSSR variation was due to differences among the four species, 74% was attributable to the within-population component, while 6% was attributable to differences among populations within species (Table 3).

Finally, we found that the seven individuals with an identical haplotype in *A. nebrodensis* showed a clear tendency to be geographically grouped within the narrow range of the population. Their locations are indicated by triangles on the map in Fig. 1.

Discussion

We have used six chloroplast microsatellites (cpSSRs) to study population genetic structure in *A. nebrodensis* and the three neighboring *Abies* species: *A. alba*, *A. cephalonica* and *A. numidica*. The high resolving power of the cpSSR analysis allowed the level of cpDNA variation within the species and the populations investigated to be quantified.

Diversity values based on haplotype frequency in the eight *Abies* populations were generally high compared to corresponding values reported for other conifer species (Powell et al. 1995; Echt et al. 1998; Morgante et al. 1998; Provan et al. 1998; Vendramin et al. 1998) and much higher than corresponding values obtained by allozyme analyses done on the same taxa (Parducci et al. 1996; Scaltsoyannes et al. 1999). These results, together with the high percentage of unique haplotypes identified, confirmed the power of cpSSR haplotype analysis for identifying individual trees in individual populations.

Based on all measures of haplotype variation, a decreased level of variation was observed in *A. nebrodensis* when compared to the other three species investigated. We also found relatively low values of genetic distance

among individuals and a low value of the "proportion distinguishable" in *A. numidica*. It is likely that prolonged isolation and small population size are the main causes of this situation in the two species. In contrast, based on all measures *A. cephalonica* was found to be the most variable species, supporting the results from several allozyme studies indicating that genetic variability in this species is high compared to other species of the same genus (Fady and Conkle 1993; Scaltsoyiannes et al. 1999; Parducci 2000, submitted).

We found 18 different alleles at the locus Pt30204. The known sequence of this locus in other conifer species revealed only two mono-nucleotide [$d(C)_n$ and $d(T)_n$] stretches (Wakasugi et al. 1994; Vendramin et al 1996). In contrast, sequencing analysis of this locus in two *Abies* species revealed that it consists of three different mono-nucleotide stretches [$d(A)_n$, $d(C)_n$ and $d(T)_n$] and differences in size are not only due to differences in the number of repeats but also to insertions/deletions of flanking and interspersed non-repetitive sequences (Vendramin and Ziegenhagen 1997). As already pointed out by Vendramin et al. (1999), it appears that in *Abies* this locus is a mutational hot spot characterized by a higher mutation rate compared to other cpSSR loci.

A. nebrodensis versus other species

Estimates of the effective number of haplotypes and haplotype diversity based on six cpSSR loci showed that *A. nebrodensis* was the least-variable of the eight *Abies* populations investigated. In contrast, previous allozyme studies did not show a significantly lower level of variation in *A. nebrodensis* compared to other *Abies* species (Vicario et al. 1995; Ducci et al. 1999; Parducci 2000, submitted). The haploid cpDNA is more-sensitive than nuclear DNA to reductions in the number of individuals in a population because its effective population size is half that of the nuclear DNA (Birky 1988). Therefore, our results from the cpSSR analysis suggest that *A. nebrodensis* experienced a genetic bottleneck in the past, as a result of a severe reduction in population size, and consequent loss of genetic diversity. Our finding that 41% of the individuals had an identical cpSSR haplotype in the population supports this hypothesis and suggests also that the variation in the chloroplast gene pool of the extant individuals is rather limited and might be further reduced in the future. In our opinion, it is unlikely when analyzing six cpSSR loci, that two identical haplotypes are produced independently by chance in individuals of the same population, i.e. that homoplasy would occur. Therefore, we can assume with confidence that the *A. nebrodensis* trees with identical haplotypes had the same, or at least a reduced, number of paternal parents. Their tendency to group in the same area and the low value of D^2_{sh} observed in the population further support this suggestion.

Our results agree with the information available on the history of this species. According to Morandini et al. (1994), the decline of *A. nebrodensis* occurred in relatively recent times and several authors attested to the existence of extensive *Abies* forests in the Madonie Range until approximately 200 years ago (Morandini et al. 1994, and references therein). In the following two centuries these forests were almost completely destroyed by man. By the beginning of the 19th century *A. nebrodensis* was considered to be lost by the scientific community, although later investigations in the 1930 s led to the discovery of a few individuals in a restricted area of the Madonie Range. These individuals, together with those discovered in the following years, constitute the extant *A. nebrodensis* population and are, therefore, at least 70–80 years old. It is likely that during the last two centuries, due to the reduction in population size, many alleles were lost in this species. Consequently, only a fraction of the original genetic variation of the species was present in the small number of founders that gave rise to the extant population (founder effect). At the same time, the few available mates and the lack of contact with other heterogeneous sources of variation increased the level of relatedness among the remaining individuals (inbreeding), and the further reduction in genetic variation was probably also accompanied by genetic drift. The high percentage of individuals with identical haplotypes found in the extant *A. nebrodensis* population, and the high level of relatedness observed among the individuals analyzed in this study, confirm our suggestion. An indication of a high level of relatedness among the *A. nebrodensis* individuals was also recently observed with allozymes by Ducci et al. (1999).

However, as already pointed out by other authors (Vicario et al. 1995; Ducci et al. 1999), despite the extremely small population size of *A. nebrodensis*, it seems that the few individuals left in this species still retain a considerable amount of the original genetic variation. Therefore, in our opinion, special attention should be given to the preservation of these individuals and to their propagation for ex situ preservation. Results from the present study may be useful for the selection of the most-unrelated individuals for the establishment of artificial stands outside the Madonie Range in order to maximize the genetic diversity of this species.

Intra-specific differentiation

We found that as many as six different haplotypes were shared among the four *A. cephalonica* populations investigated. This result was concordant with the absence of population differentiation observed in this species with the AMOVA analysis (F_{ST}=0.012), and suggests that gene flow was active among the populations of this species. In contrast, we found no common haplotypes between the two populations of *A. alba* studied and results from the AMOVA analysis showed a significant level of population differentiation (F_{ST}=0.191). Similar results were recently obtained in *A. alba* by Vendramin et al. (1999). Using two of the six cpSSR loci analyzed in this study (Pt30204 and Pt71936), the

authors found a high level of population differentiation among several populations collected all over the *A. alba* range (G_{ST}=0.133).

The different results in the level of apportionment of genetic variation for the two *Abies* species were not entirely surprising, and can be explained if we hypothesize that, unlike *A. cephalonica*, the extant European *A. alba* populations had multiple post-glacial origins. It is believed that during the Miocene (26–5 My BP) an ancient *Abies* progenitor existed in the Balkan Peninsula (Liu 1971; Huntley and Birks 1983). During the successive warm periods of the Tertiary (65–1.6 My BP) this progenitor differentiated into many different *Abies* species, which became widely distributed in the Northern Hemisphere. During the last ice age (90000–12000 BP) the differentiated *Abies* species withdrew southwards and into lower elevations, and in Europe they were restricted basically to three refugia: in Calabria, the Balkans and south Iberia. The occurrence of such refugia is now well-documented (Huntley and Birks 1983) and their detection is crucial to the understanding of the contrasting patterns of genetic variation observed in the current *Abies* species. After the melting of the glaciers (12000–6000 BP) *A. alba* moved northwards from the Balkan refugium to re-colonize all of central Europe and *A. cephalonica* remained confined to central and southern Greece. In addition, *A. alba* followed two other migration routes: one from the Calabrian refugium to central Italy and one from the Iberian Peninsula to central Europe. By 10000 BP, *A. alba* was present in the Alps and subsequently expanded steadily in this area.

The different postulated post-glacial origins of the European *A. alba* populations agree with the significant level of differentiation observed among the two *A. alba* populations investigated in this study, as well as with the results obtained by Vendramin et al. (1999). Similarly, the evidence that different migrants merged from several refugia supports our hypothesis of a multiple origin for the *A. alba* populations occurring in northern and central Europe, and is consistent with the higher level of genetic distance observed among the individuals of population ALBA-1 compared to ALBA-2. In contrast, the unique post-glacial migration of *A. cephalonica* from the Balkan refugium, and the likely confinement of this species to the Greek Peninsula, probably promoted intensive gene exchange among the neighboring populations. We believe this postulated intensive gene exchange was the main cause of the very low level of differentiation observed among the four *A. cephalonica* populations analyzed in this study.

We found a high level of differentiation among the D^2_{sh} values observed in the eight *Abies* populations. With the sole exception of population CEPE, we found that the D^2_{sh} values were related with the size and the type of location of the population under study. Low D^2_{sh} values were observed in *A. nebrodensis* and *A. numidica*, both of which have small and isolated ranges. They were also found in population CEKE from Cephalonia Island, in a region completely isolated from the main distribution center of *A. cephalonica*, and in population ALBA-2, located in the Calabria region, that is very distant from the main distribution center of *A. alba* in central Europe. As already noted, the lack of contact with other heterogeneous sources of variation and, in some cases the small population size, are likely to be the main causes of the increased level of relatedness observed among individuals of these populations.

Finally, we found high "proportion distinguishable" values in at least five out of eight populations analyzed. An unexpected result was obtained in population ALBA-1 where, although all the individuals were clearly differentiated from each other, only 52% of them could be genotypically identified. This result can be explained if we assume that the number of fathers (pollinators) recently decreased in this population. Such a reduction in the number of pollinators would increase the number of individuals with identical haplotypes in a few generations, but would not affect the overall level of genetic variation of the population so rapidly (H_e=0.933). Low vitality and pronounced signs of decline have been observed in *A. alba* individuals in recent decades in the northern parts of its natural distribution (Bergmann et al. 1990). It is possible that his decline is strongly linked to the reduction in the number of fathers in the population.

Inter-specific differentiation

Our findings, showing a lack of common haplotypes between *A. nebrodensis* and the other populations, provided no evidence of common ancestry or past-hybridization between this species and the other *Abies* species analyzed in this study. These results are in agreement with previous studies showing that *A. nebrodensis* is differentiated from both *A. alba* and *A. cephalonica* (Vicario et al. 1995; Ducci et al. 1999; Parducci and Szmidt 1999), and support its classification as an independent species.

We found two haplotypes in common between the populations ALBA-2 and CEPE, and one haplotype in common between ALBA-2 and *A. numidica*. As already mentioned, the CEPE population occurs in central Greece, in a region where the ranges of *A. alba* and *A. cephalonica* overlap. Therefore, our results seem to agree with the hypothesis that a past genetic contact occurred in this region between the two species and with the presence of hybrid *Abies* populations (*A. borisii-regis*). However, further analyses employing specific diagnostic markers for the two parental species should be carried out to further clarify the taxonomy of the *Abies* populations occurring in this region. On the other hand, the finding of a common haplotype between population ALBA-2 and *A. numidica* was more difficult to interpret and suggest that the two species may have come in contact some time in the post.

Evidence reported in this paper may be considered a useful contribution to a better-understanding of the history of *A. nebrodensis*. We also showed that cpSSR polymorphism is useful to analyze variation within different *Abies* species and that cpSSR haplotypes can identify individual trees in individual populations.

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